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Crystallographic Studies of the Interaction of Cyclodextrin Glycosyltransferase from *Bacillus circulans* Strain 251 with Natural Substrates and Products*

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Asp-229, Glu-257, and Asp-328 constitute the catalytic residues in cyclodextrin glycosyl transferase from *Bacillus circulans* strain 251. Via site-directed mutagenesis constructed D229N, E257Q, and D328N mutant proteins showed a 4,000–60,000-fold reduction of cyclization activity. A D229N/E257Q double mutant showed a 700,000-fold reduction and was crystallized for use in soaking experiments with α-cyclodextrin. Crystal structures were determined of wild type CGTase soaked at elevated pH with α-cyclodextrin (resolution, 2.1 Å) and maltoheptaose (2.4 Å). In addition, structures at cryogenic temperature were solved of the unliganded enzyme (2.2 Å) and of the D229N/E257Q mutant after soaking with α-cyclodextrin (2.6 Å). In the crystals soaked in α-cyclodextrin and maltoheptaose, a maltotetraose molecule is observed to bind in the active site. Residue 229 is at hydrogen bonding distance from the C-6 hydroxyl group of the sugar, which after cleavage will contain the new reducing end. In the D229N/E257Q double mutant structure, two α-cyclodextrins are observed to replace two maltoses at the E-domain, thus providing structural information on product inhibition via binding to the enzyme's raw starch binding domain.

Cyclodextrin glycosyltransferases (CGTases; E.C. 2.4.1.19) are monomeric bacterial enzymes that catalyze the conversion of starch into cyclic or linear α(1→4)-linked glucopyranosyl chains (1). In general, CGTases produce mixtures of cyclic compounds named cyclodextrins (CDs),[1] consisting of six, seven, or eight D-glucopyranose units, which are referred to as α-, β-, and γ-cyclodextrins, respectively. Depending on the major type of cyclodextrin that is produced, CGTases are classified as α-, β-, or γ-CGTases. The β-CGTase from *Bacillus circulans* strain 251 is one of the CGTases that is currently used for the industrial production of β-cyclodextrins. This process is, however, hampered by the sensitivity of the enzyme to product inhibition and by its relatively low product specificity. We investigate this enzyme with the aim of gaining more insight into the factors that determine its functionality, in order to guide the rational design of mutants with improved properties.

Recently, we reported the nucleotide sequence and crystal structure at 2.0 Å resolution of this CGTase (2) and its complex with the inhibitor acarbose (3). The enzyme consists of 686 amino acids grouped in five distinct domains, labeled A through E. Domains A, B, and C are structurally homologous to the equivalent domains of α-amylases. The E-domain has been implicated in starch binding (4) and was found to bind two maltose molecules. A third maltose molecule is bound by the C-domain and is involved in crystal packing contacts between symmetry related molecules. On the basis of the structure of the enzyme complexed with acarbose, it was concluded that Glu-257 acts as the proton donor in the reaction, whereas Asp-229 serves as the general base or nucleophile. Asp-328 is involved in binding of the substrate and helps to elevate the pK_a of Glu-257 through a direct hydrogen bond to this residue (2, 3) that exists only when no substrate or inhibitor is present. Surprisingly, the acarbose inhibitor was not cleaved, even though this maltotetraose was observed to bind near the catalytic residues Glu-257 and Asp-229 with the *O*-glycosidic bond between the B and C sugar residues, in a seemingly productive way. As a possible explanation it was suggested that the presence of a hydroxyl group at the C-6 carbon atom of the B sugar is essential for either the destabilization of the conformation of the β-sugar or for the polarization of the atoms of the scissile glycosidic bond. Structural data on the interaction of CGTase with natural substrates, which do possess a hydroxyl group at the C-6 atom of the β-sugar, could clarify this aspect of the catalytic mechanism of CGTases.

Although the crystallization at pH 7.55 of CGTase from *B. circulans* strain 251 requires the presence of α-CD or maltose (5), no oligosaccharides were observed to bind near the active site carboxylates (2). Soaking of crystals of a D229A mutant of the highly similar *B. circulans* strain 8 CGTase (6) at pH 6.7 in a β-cyclodextrin solution revealed only the presence of a single maltose residue in the active site. These experiments were, however, hindered by the instability of the crystals in the presence of oligosaccharides, requiring the use of extremely short soaking periods (7). Therefore, to establish the binding mode of native substrates in the active site cleft, experiments should ideally be performed under conditions where the enzyme has no or very low activity. As the catalytic mechanism of CGTases and α-amylases is thought to be similar to that of lysozyme (3, 8) and as the first step of the reaction entails

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The atomic coordinates and structure factors (codes 1cxe, 1cxf, 1cxh, and 1cxi) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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[1] The abbreviations used are: CD, cyclodextrin; CGTase, cyclodextrin glycosyl transferase; G7, maltoheptaose; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; r.m.s., root mean square.

donation of a proton by the catalytic glutamic acid residue (Glu-257 in the case of CGTase) to the glycosidic oxygen of the scissile bond (3, 9), the rate of the reaction may be significantly reduced by raising the pH of the soaking solutions. A completely different approach involves the inactivation of catalytic residues Asp-229, Asp-328, and Glu-257 by site-directed mutagenesis. Here we report the preparation, purification, and determination of the catalytic properties of such mutants, as well as the crystal structures of enzyme-substrate complexes obtained by soaking crystals of wild type and a D229N/E257Q double mutant CGTase in solutions of α-cyclodextrin or maltoheptaose (G7) at elevated pH.

MATERIALS AND METHODS

Bacterial Strains, Bacteriophage, and Plasmids—*Escherichia coli* MC1061 (*hsd*R *mcr*B *ara*D139 D(*ara*ABC-*leu*)7679 D*lac*X74 *gal*U *gal*K *rps*L *thi*) (10) was used for recombinant DNA manipulations. *E. coli* CJ236 (*dut*1 *ung*1 *thi*-1 *rel*A1/pCJ105 (Cm^r F′)) (11) was used for site-directed mutagenesis. CGTase (mutant) proteins were produced with the α-amylase and protease negative *Bacillus subtilis* strain DB104A (*amy his npr*R2 *npr*E18 *apr*A3) (12). The bacteriophage M13K07 was used for preparing single-stranded DNA (13).

Growth Conditions—Plasmid-carrying bacterial strains were grown on LB medium in the presence of the antibiotics ampicillin (plasmid pDV58) (2), erythromycin and spectinomycin (pDP66S) (14), or erythromycin and kanamycin (pDP66K)[2] at concentrations of 100 and 5 μg/ml for *E. coli* and *B. subtilis*, respectively (15). When appropriate, agar plates contained 1% starch to screen for halo formation. *B. subtilis* strain DB104A was grown in a 1.5–3-liter batch fermenter as described previously (14).

DNA Manipulations—Restriction endonucleases and Klenow enzyme were purchased from Pharmacia LKB Biotechnology and used according to the manufacturer's instructions. DNA manipulations and calcium chloride transformation of *E. coli* strains were as described (15). Transformation of *B. subtilis* was performed according to Bron (16).

Site-directed Mutagenesis—To construct single amino acid mutations the method described by Kunkel *et al.* (11) was used. Single-stranded DNA was prepared using plasmid pDV58 and *E. coli* strain CJ236 after infection with bacteriophage M13K07. Three oligonucleotides were used to produce the mutations GGCATCCGCATGAATGCGGTGAAGCAT for D229N, ATCGATAACCATAACATGGAGCGT for D328N, and CGGTCTTTACCTTTGGCCAATGGTTCCTGGGC for E257Q. Successful mutagenesis also resulted in the appearance of the underlined restriction sites, allowing rapid screening of presumptive mutants. For D229N this restriction site was *Bsm*I, for D328N it was *Cla*I, and for E257Q it was *Bal*I. After mutagenesis the DNA was transformed to calcium chloride competent *E. coli* MC1061 cells. For the construction of the double mutation (D229N/E257Q) mutant D229N DNA was subcloned in pDP66S (14) (*Pvu*II–*Nar* I fragment) and used as a template for polymerase chain reaction mutagenesis[2] using VENT-DNA polymerase (New England Biolabs, Beverly, MA) with primer E257Q.

Production and Purification of CGTase (Mutant) Proteins—Plasmid pDV58 carrying positively characterized single mutant *cgt* genes and plasmid pDP66S (14) was digested with *Pvu*II and *Nar*I. The 1207-base pair fragment from the expression vector pDP66S was replaced with the corresponding fragment containing the mutation from the mutagenesis vector pDV58, ligated, and transformed to *E. coli* strain MC1061. For the double mutant the polymerase chain reaction product was cut with *Pvu*II and *Nar* I as well and exchanged for the corresponding fragment from plasmid pDP66K.[2] After isolation of pDP66S and pDP66K DNA and restriction analysis, the plasmid DNA was transformed to *B. subtilis* strain DB104A. The organism was grown to an optical density of 13 at 600 nm in a 1.5–3-liter batch fermenter (for approximately 50 h). Every 12 h additional erythromycin (10 μg/ml) was added to the medium. Under these conditions high extracellular CGTase levels were produced. The culture was centrifuged at 4 °C for 30 min at 16,000 × *g*. The supernatant proteins were further purified to homogeneity by affinity chromatography, using a 30-ml α-cyclodextrin-Sepharose 6FF column (Pharmacia) (17) with a maximum capacity of 3.5 mg of protein/ml. After washing with 10 mM sodium acetate buffer (pH 5.5), bound CGTase was eluted with the same buffer containing 10 mg/ml α-cyclodextrin.

Enzyme Assay—The β-cyclodextrin-forming activity was measured as described previously (14) by incubating appropriately diluted enzyme with 5% Paselli SA2 as a substrate dissolved in 10 mM sodium citrate buffer (pH 6.0). The amount of β-cyclodextrin formed was determined on the basis of its ability to form a stable, colorless inclusion complex with phenolphthalein (18). One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin/min.

Determination of the pH Optima—In order to define the pH optima for wild-type CGTase and the three single active site mutants appropriate dilutions of enzyme were incubated in a 5% Paselli solution buffered with a 10 mM sodium citrate/Hepes buffer with a pH ranging from 2 to 11. After 3 min the reactions were stopped and the amount of β-cyclodextrin was measured using phenolphthalein.

Crystallization, Soaking, and Data Collection—Wild-type and mutant CGTases from *B. circulans* strain 251 were crystallized as described by Lawson *et al.* (5). The cell dimensions are listed in Table I.

For the soaking experiments with α-cyclodextrin, the crystallization mother liquor was replaced by a soaking solution composed of 60% (v/v) 2-methyl 2,4-pentanediol, 100 mM CAPS buffer, pH 9.1, and 0.5% of α-cyclodextrin (w/v). In the case of the double mutant CGTase crystals, a 1% α-cyclodextrin concentration was used. For the soaking experiments with maltoheptaose, the pH of the mother liquor was increased to pH 9.6, and 0.5% maltoheptaose was added to the buffer. All soaking experiments were carried out at room temperature. The wild-type enzyme was soaked in the substrate solutions for 5 days, whereas the double mutant was soaked for 1 h.

The double mutant and unliganded native enzyme crystals were flash frozen to 120 °K using 2-methyl 2,4-pentanediol as cryoprotectant (19) in order to reduce any residual catalytic activity and to limit radiation damage. The other two data sets (Table I) were recorded at room temperature. All data were obtained from a single crystal per data set on an Enraf Nonius FAST area detector system mounted on an Elliot GX21 rotating anode generator as the x-ray source. Data collection and processing was done with MADNES (20) with profile fitting of the intensities according to Kabsch (21). A summary of data collection statistics is listed in Table I.

Structure Refinement—Refinement of the crystal structures was done with the TNT package (22) using the 2.0 Å structure of native CGTase (2) as the starting model for rigid body and subsequent all parameter refinement. This model consisted of all 686 residues, two calcium ions, and three maltose molecules. Ideal bond lengths and angles were taken from Engh and Huber (23). Free crystallographic *R*-factors (24) were calculated using a subset of 10% randomly selected reflections. In the case of wild-type enzyme complexed with α-CD and G7, free *R*-factors were only calculated during the last 10 cycles of refinement, whereas for the other structures free *R*-factors were monitored during the entire refinement. Adjustments to models were made using FRODO (25) running on an Evans and Sutherland PS390 computer graphics station and O (26) running on Silicon Graphics workstations on the basis of σ_a-weighted ($2\,m|F_o| - D|F_c|$) and ($m|F_o| - D|F_c|$) difference maps (27). Water molecules were only included in the structure if the corresponding peak in the difference electron density map exceeded 3.5 σ and the water molecule could form at least one hydrogen bond with a protein atom or with an already existing water molecule. Water molecules at more than 3.3 Å from protein atoms or previously placed water molecules were eliminated from the model. All water molecules were visually inspected using FRODO or O. Starting temperature factors were taken from the native protein model or set to 20 Å^2 for newly added ligands or water molecules. Refinement statistics are summarized in Table I.

During and after completion of the refinement, the final models were analyzed with the PROCHECK package (28) and the program OOPS (29). Models for CGTase complexed with maltotetraose and α-CD, as well as that of the native protein and the D229N/E257Q mutant determined at 120 °K, have been deposited with the Protein Data Bank, Brookhaven National Laboratory (30) (entries 1cxe, 1cxf, 1cxh, and 1cxi, respectively).

RESULTS

Characteristics of the Mutant Enzymes—A single fermenter run with *B. subtilis* DB104A allowed purification of up to 300 mg of (mutant) CGTase protein in a 60–70% yield. Whereas no cyclodextrin-forming activities could be found in crude extracts of strains carrying the D229N, D328N, E257Q, and D229N/E257Q mutations, expression of these mutant CGTase proteins

[2] D. Penninga, B. A. van der Veen, R. M. A. Knegtel, S. A. F. T. Van Hijum, H. J. Rozeboom, K. H. Kalk, B. W. Dijkstra, and L. Dijkhuizen, manuscript in preparation.

TABLE I
Data collection statistics and quality of the final models

Data collection and refinement statistics are given for the wild-type (wtCGTase) and D229N/E257Q double mutant CGTase structures based on crystals soaked with α-CD or G7. The R_{merge} is defined as: $R_{merge} = (\Sigma\Sigma \left| Ij(hkl) - \langle I(hkl)\rangle \right|)/(\Sigma\Sigma \langle I(hkl)\rangle)$, and the crystallographic *R*-factor is defined as: $R = \Sigma \left| |F_o| - |F_c| \right| / \Sigma |F_o|$. Free *R*-factors were calculated using 10% of the unique reflections.

Protein	wtCGTase	wtCGTase	wtCGTase	D229N/E257Q
Soaked with	α-CD	G7		α-CD
Temperature (K)	293	293	120	120
Cell dimensions ($P2_12_12_1$)				
a (Å)	121.4	120.5	118.0	116.8
b (Å)	111.6	111.3	109.8	110.0
c (Å)	66.4	66.0	65.7	67.4
Resolution range (Å)	40.2–2.1	40.8–2.4	9.6–2.2	40.8–2.6
Total number of observations	54298	35372	186267	40543
No. of discarded observations	9850	4816	5854	701
No. of unique reflections	44448	30556	39669	21240
R_{merge}	0.051	0.069	0.087	0.054
Completeness of data (%)	82.9	86.7	89.7	79.3
Completeness of last (%)	36.1	14.3	79.3	13.1
Resolution shell (Å)	2.13–2.10	2.45–2.41	2.23–2.20	2.67–2.62
No. of protein atoms	5264	5264	5264	5264
No. of calcium atoms	2	2	2	2
No. of carbohydrate atoms	114	114	69	200
No. of solvent sites	417	195	468	310
Average B-factor (Å^2)	22.9	20.4	12.1	10.5
Final *R*-factor/*R* free	15.3/19.0	18.0/23.4	18.0/24.3	19.0/28.6
r.m.s. deviations from ideality				
Bond lengths (Å)	0.007	0.006	0.008	0.009
Bond angles (deg)	1.1	1.1	1.3	1.1
Torsion angles (deg)	17.5	17.9	17.1	18.6
Trigonal planes (Å)	0.008	0.009	0.009	0.009
Planar groups (Å)	0.010	0.010	0.013	0.010
van der Waals contacts (Å)	0.018	0.021	0.015	0.016
r.m.s. difference in B for neighboring atoms (Å^2)	2.18	2.66	1.26	0.96

could clearly be detected by an enzyme-linked immunosorbent assay. Analysis of purified mutant proteins showed that they had almost completely lost their cyclodextrin-forming activity (4,000–700,000-fold reduction; see Table II). Because the double mutant CGTase had the lowest catalytic activity, it was selected for substrate binding studies. As can be seen in Fig. 1, the pH optima of the mutants E257Q and D328N have shifted downwards by 0.5 unit compared with the wild-type enzyme. The optimum of mutant D229N remained at pH 6.0, the value of the wild-type enzyme. Interestingly, the wild-type enzyme was still partially active at pH 9 (Fig. 1*A*). Most likely the single and double mutants have residual activity at elevated pH as well because minor activity changes were observed with increasing pH values (Fig. 1*B*).

Structure Refinement—In order to analyze the interaction of CGTase with substrates, x-ray structures were determined at room temperature (CGTase complexed with α-cyclodextrin or maltoheptaose) and at cryogenic temperature (120 °K; wild-type CGTase and the D229N/E257Q mutant complexed with α-cyclodextrin). Table I presents a summary of the final results of the crystallographic refinement. Possibly due to differences in completeness and resolution between the data sets, structures refined against data obtained at higher resolution yield slightly lower crystallographic *R*-factors, which in addition are closer to the corresponding free *R*-factors.

Ramachandran plots (31) of CGTase with bound oligosaccharides are virtually identical to that of native CGTase (2). Comparison of native and complexed structures yields r.m.s. differences in C_α coordinates of 0.3–0.4 Å, which are of the same magnitude as the mean coordinate error of 0.2 Å as derived from σ_a plots (27). No large structural rearrangements are observed due to ligand binding or the use of flash freezing. A comparison of the C_α coordinates of the uncomplexed wild-type structures determined at 293 and 120 °K yielded a r.m.s. difference of 0.3 Å, which indicates that measuring at cryogenic temperature also does not induce substantial conformational

TABLE II
β-Cyclodextrin-forming activities of B. circulans strain 251 wild-type and mutant CGTase proteins

One unit of activity is defined as the amount of enzyme able to produce 1 μmol of β-cyclodextrin/min.

Protein	Activity
	units/mg
Wild type	280 ± 5
D229N	0.012 ± 0.002
E257Q	0.067 ± 0.005
D328N	0.005 ± 0.001
D229N/E257Q	0.0004 ± 0.0001

changes. Only small changes in the protein backbone conformation of surface loops near the active site and the maltose-binding sites are observed in all cases. Binding of α-CD to the E-domain induces relatively the largest changes. Structures refined with data recorded at cryogenic temperature have average B values for protein atoms in the order of 10 Å^2, which is about half the magnitude of those of structures refined with data recorded at room temperature, indicating an overall decrease in flexibility.

After completion of the refinement it appeared that the soaking experiments with α-cyclodextrin at pH 9.1 as well as those with maltoheptaose at pH 9.6 had produced a very similar electron density in the active site, consistent with that for a maltotetraose molecule. Apparently even at high pH values the enzyme still has sufficient residual activity to partially hydrolyze substrates (*cf.* Fig. 1). Even the experiment with the D229N/E257Q double mutant at cryogenic temperature (120 °K) showed only electron density corresponding to a maltotetraose in the active site. The density for maltotetraose is not unambiguous for all atoms. The electron density that was observed in OMIT maps (32) in the active site was, however, of a similar shape in all three complex structures and overlapped well with the conformation of acarbose in complex with CGTase

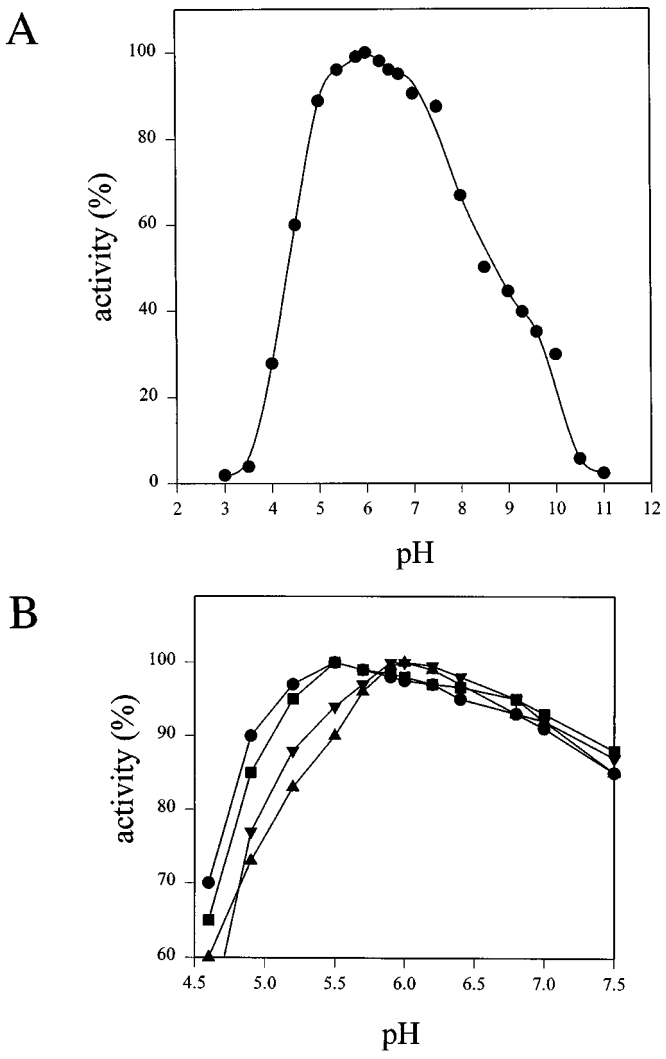

FIG. 1. **pH optima for the wild-type and active site mutants D229N, E257Q, and D328N of *B. circulans* strain 251 CGTase.** In *A* the pH profile of wild-type CGTase is shown. β-Cyclodextrin-producing activity was measured using a 10 mM citrate/Hepes buffer at 50 °C. *B* shows a comparison of the pH profiles near the pH optima for wild-type CGTase ( ) and the active site mutants D229N ( ), E257Q (■), and D328N (●).

(3). The hydroxymethyl groups of the maltotetraose molecule in the double mutant structure were well enough defined in the electron density of the A and C sugars to allow unambiguous identification of the direction of the polysaccharide chain. The carbohydrate residues have high temperature factors (55–65 Å^2) compared with an average B-factor of about 20 Å^2 for the surrounding protein atoms. This suggests a low occupancy or the binding of maltotetraose in multiple (distorted) conformations. In the case of the CGTase of *B. circulans* strain 8, soaking of protein crystals with β-cyclodextrin (7) yielded electron density of a similar quality for a maltose molecule in the active site. The average value for the B-factors of about 55 Å^2 suggests that glucoses B and C interact more strongly with the residues of the active site than the A and D residues (which have B-factors in the order of 65 Å^2) as was also observed for acarbose complexed with CGTase (3). No significant differences were found between the conformations of maltotetraose in the three complexes. The r.m.s. differences in coordinate positions for the maltotetraose atoms are in the order of 0.4–0.6 Å and are mainly due to different orientations of O-6 hydroxyl groups. High B-factors of the maltotetraose atoms and the imperfect electron density prevent the accurate determination of deviations from a full $^4C_1$ chair conformation of the glucose subunits.

Binding of Maltotetraose in the Active Site of CGTase—The mode of binding observed for the maltotetraose molecule bound in the active site cleft of CGTase resembles closely that observed for acarbose (3), where a sharp turn between the sugar residues at subsites B and C was induced upon binding. This conformation of maltotetraose agrees with the proposed role of acarbose as a substrate analog rather than a transition state analog (3). Figs. 2 and 3 depict the maltotetraose molecules and their $2F_o - F_c$ electron densities in the various structures solved. Like in the acarbose structure (3), the maltotetraose structure consists of two highly similar canonical maltose molecules (residues A-B and C-D), linked together through an unusual glycosidic bond formed by the B-C linkage, which lacks the internal hydrogen bond between the O-2B and O-3C hydroxyl groups. This twisted mode of substrate binding closely resembles the ligand conformation observed in the complex between pig pancreatic α-amylase and an inhibitor (33). Also in this case a sharp turn in the substrate is observed at the scissile glycosidic bond.

Table III lists possible hydrogen bonds between maltotetraose and CGTase in the complex structures. For comparison the equivalent contacts observed in the complex with acarbose are also listed. From Table III it is clear that most intermolecular contacts observed in the complex between CGTase and acarbose (3) are conserved in the complex with maltotetraose. A number of interesting differences are observed, however, which will be discussed in the following paragraphs.

In the complexes between CGTase and maltotetraose, Asp-229 forms a strong hydrogen bond with the C-6 hydroxyl group of sugar B, which is absent in acarbose. Although Asp-229 is now involved in a hydrogen bond with sugar B, its conformation has not changed from that observed in the complex with acarbose. The second oxygen atom of the carboxylate group of Asp-229 is at 2.8–3.0 Å of the C-1 atom of glucose B and could stabilize a positively charged oxo-carbonium intermediate. The conformations of the side chains of Asn-229 and Gln-257 in the double mutant CGTase complexed with maltotetraose are very similar to those observed in complexes of the native protein. Additional stabilization of the sugar ring at subsite B comes from stacking interactions with the aromatic ring of Tyr-100. Unfortunately the density around the sugar at subsite B is of poor quality in all three complexes. Possibly this sugar assumes a number of different distorted conformations, yielding an averaged and decreased electron density. Except for the complex with G7, the electron density covers the glycosidic linkage between the B and C sugars, indicating that the majority of the bound maltotetraoses is uncleaved (cf. Fig. 2).

Tyr-195 seems to interact only weakly with sugar C of maltotetraose. In the high pH complexes of CGTase with maltotetraose, the distance between its hydroxyl group and the O-6C hydroxyl group is roughly 3.8 Å. Nevertheless, the O-6C hydroxyl group could easily interact with the Tyr-195 hydroxyl group after rotation about the C-5C-C-6C bond. Such a conformation is actually observed in the double mutant-maltotetraose complex where the Tyr-195 hydroxyl group is only 2.7 Å away from the O-6C hydroxyl group. In the other two complexes the O-6C hydroxyl groups are near and could be directed toward the Tyr-195 hydroxyl group as well. Tyr-195 has been implicated in the product specificity of CGTases, although the precise role of this residue is not known (14, 34). Perhaps the impossibility of forming this hydrogen bond causes the decrease in cyclization and coupling activity observed for a Y195F mutant (14).

An interesting difference in the side chain conformation of Lys-232 is observed in the structure derived from data obtained

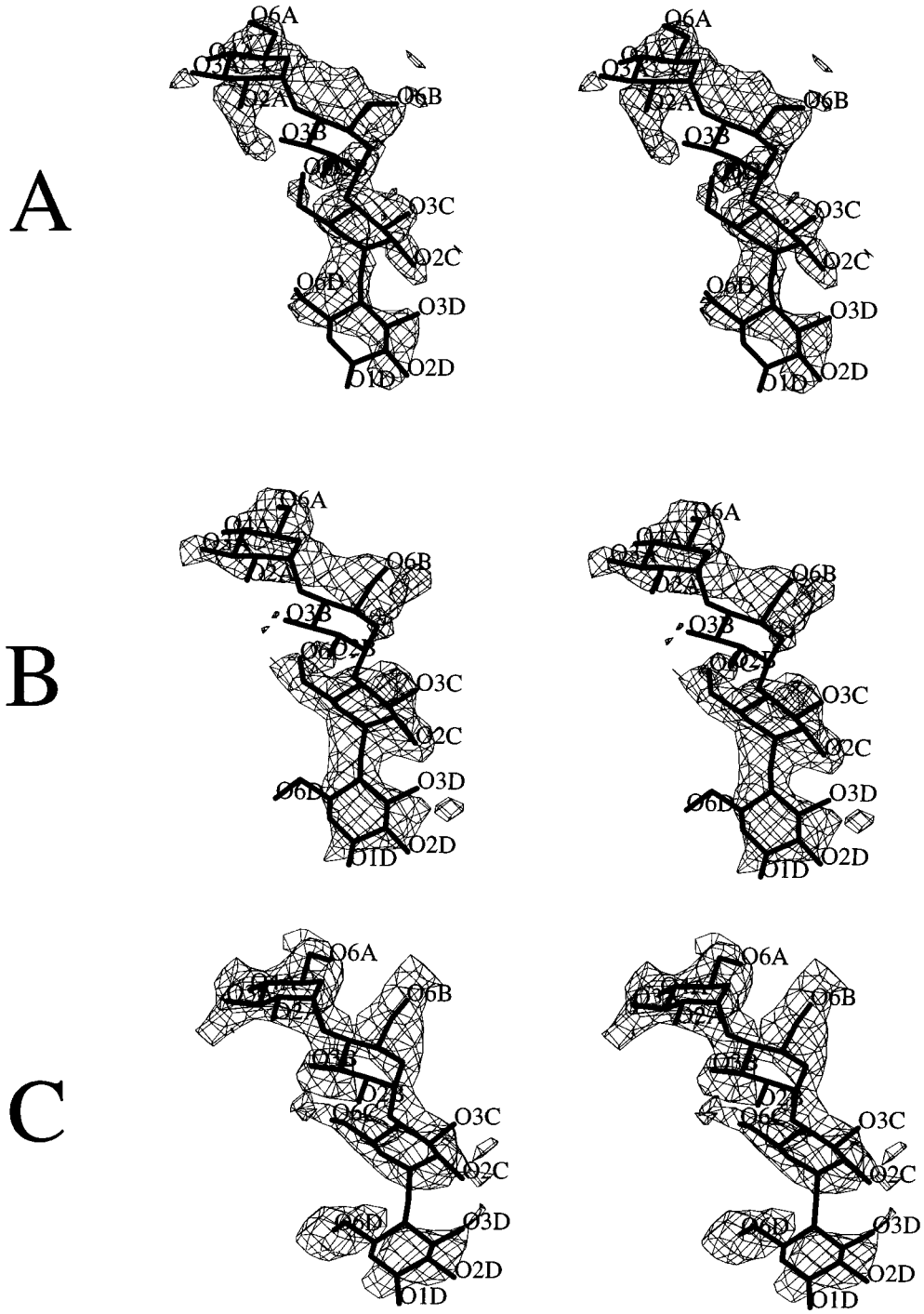

FIG. 2. **Stereo views of the electron density observed for bound maltotetraose in the active site of CGTase.** Shown are the maltotetraose molecules in the structures of CGTase (*A*) soaked with α-CD, CGTase soaked with G7 (*B*), and D229N/E257Q CGTase soaked with α-CD (*C*). Electron density in $2F_o - F_c$ maps is contoured at 1 σ, and hydroxyl groups of the maltotetraose molecule are labeled.

for CGTase crystals soaked with maltoheptaose, which results in the formation of a strong hydrogen bond between the side chain amino group and the O-2D hydroxyl atom of the ligand as illustrated in Fig. 3. This hydrogen bond is not observed in structures derived from soaking experiments with α-CD but is present in the complex with acarbose (*cf.* Table III). Although in all structures derived from crystals soaked with α-CD structural variability is observed for the Lys-232 side chain, the side chain conformations cluster in an entirely different group than the conformation observed in the CGTase-G7 complex. It seems unlikely that the relatively small change in the buffer composition from pH 9 to pH 9.6 would induce structural changes of this nature because at both pH values lysine (pK_a = 10.8) is expected to be protonated. The observed difference could be due to the fact that the initial binding of cyclodextrins and linear substrates into the active site is fundamentally different. Longer linear substrates such as amylose, maltoheptaose, or acarbose most likely enter the active site through the long groove that extends from the active site to the second maltose-binding site (2). For cyclic compounds this is not possible due to steric constraints, and they can only approach the active site directly from the solvent. Lys-232 is positioned on the border of the starch binding groove and the active site cleft, and its side chain conformation is expected to be affected by the entry of a

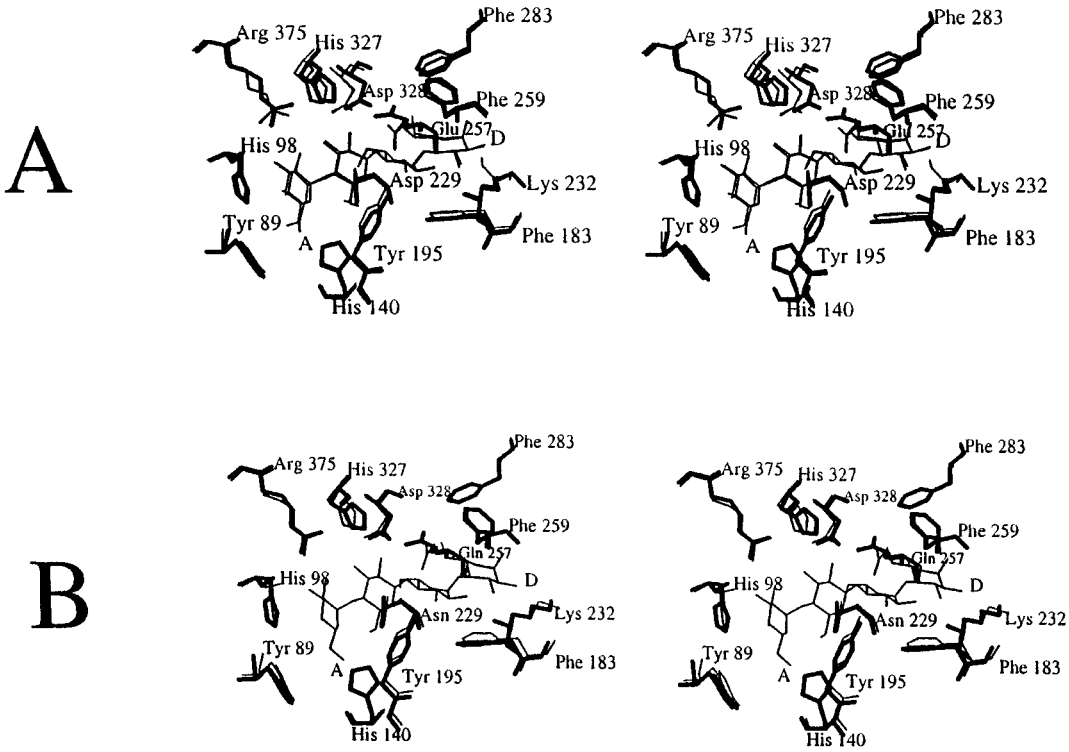

FIG. 3. **Stereo views of active site residues of (un)liganded CGTase and with bound maltotetraose.** Shown are the active site CGTase soaked with α-CD and maltoheptaose (*A*) and the active site of D229N/E257Q CGTase soaked with α-CD (*B*). Corresponding residues of the unliganded enzyme at the same temperature are drawn with *thick lines*, and the complexed protein residues and maltotetraose are drawn with *thin lines*. Residue types and sequence numbers, as well as the maltotetraose A and D sugars, are labeled.

TABLE III

Putative hydrogen bonds between maltotetraose and wild-type and mutant CGTase

Contact distances shorter than 3.5 Å are listed for structures based on crystals soaked with either α-CD or G7. For the D229N/E257Q double mutant CGTase, the Asp-229 Oδ-2 atom is to be read as Asn-229 Nδ-2, and the Glu-257 Oε-2 atom is to be read as Gln-257 Nε-2 in the table. The contacts in the complex with acarbose (3) are shown for reference. wtCGTase, wild-type CGTase.

Maltotetraose residue/atom	Protein atom or water molecule	wtCGTase + α-CD	wtCGTase + G7	D229N/ E257Q α-CD	wtCGTase + acarbose
			Å		
Residue A					
O-2A	Arg-375 Nη-1	3.2	3.0	2.7	3.3
O-2A	O-3B (internal)	2.7	2.7	2.8	3.2
O-3A	Asp-371 Oδ-2	2.9	2.8	2.7	2.8
O-3A	Arg-375 Nη-2	3.4	>3.5	3.0	3.2
O-4A	Water			3.4	
Residue B					
O-1B	Glu-257 Oε-2	2.8	2.7	3.2	2.9
O-1B	Glu-257 Oε-1	3.3	3.0	3.0	3.1
O-2B	Asp-328 Oδ-2	2.7	2.7	2.8	2.7
O-2B	His-327 Nε-2	3.1	3.1	3.2	3.1
O-3B	Asp-328 Oδ-1	3.2	3.0	3.3	2.8
O-3B	O-2A (internal)	2.7	2.7	2.8	3.2
O-6B	Asp-229 Oδ-2	2.7	2.7	3.2	
Residue C					
O-2C	His-233 Nε-2	3.1	2.9	2.7	2.7
O-2C	O-3D (internal)	2.7	2.7	2.9	3.1
O-2C	Water	2.7	3.0		2.9
O-3C	Glu-257 Oε-1	2.7	2.7	3.0	2.7
O-3C	Water	3.0	3.0		3.1
O-6C	Tyr-195 OH	>3.5	>3.5	2.7	>3.5
Residue D					
O-2D	Lys-232 N-ζ	>3.5	3.3	>3.5	3.5
O-3D	Lys-232 N-ζ	>3.5	2.7	>3.5	2.6
O-3D	O-2C (internal)	2.7	2.7	2.9	3.1

longer linear substrate via the groove. One might speculate that the additional hydrogen bond donated by Lys-232 specifically increases the binding energy of linear substrates, thus shifting the reaction equilibrium toward the formation of cyclic carbohydrates from linear carbohydrates. Fig. 4 summarizes the interactions between maltotetraose and the active site residues of CGTase.

Binding of α-Cyclodextrin to Maltose-binding Sites of CGTase—Although only a maltotetraose molecule was present in the active site of the double mutant, density for intact α-CD

molecules was found at the two maltose-binding sites in the E-domain (Fig. 5). The presence of bound α-cyclodextrin at the E-domain is not surprising because Villette *et al.* (35) demonstrated earlier that this domain is capable of binding β-CDs. The maltose-binding site located in the C-domain displayed electron density for a single maltose molecule only. This maltose molecule is involved in extensive crystal packing contacts, and the corresponding maltose-binding sites might not be readily accessible for α-CD. Alternatively, it has been suggested that the third maltose-binding site could be merely a crystallographic artifact of little biological relevance (2).

The two α-CD molecules bind to CGTase with the apolar sides of the glucose units stacked on aromatic amino acid residues of the protein. Similar hydrophobic stacking interactions have been observed for β-CD bound to the maltodextrin binding protein (36) and α-CD complexed with pig pancreatic α-amylase (37) and soybean β-amylase (38). A list of possible intermolecular hydrogen bond interactions between the α-CDs and CGTase is presented in Table IV. The numbering A–F of the glucose units in α-CD is chosen such that residue A corresponds to the nonreducing sugar of the originally bound maltose (2), and residue B corresponds to the reducing sugar. The bound α-CD molecules form essentially the same set of hydrogen bonds with the enzyme as observed for the maltoses in the native structure (2). Some differences are present, however, and they will be discussed briefly in the following sections.

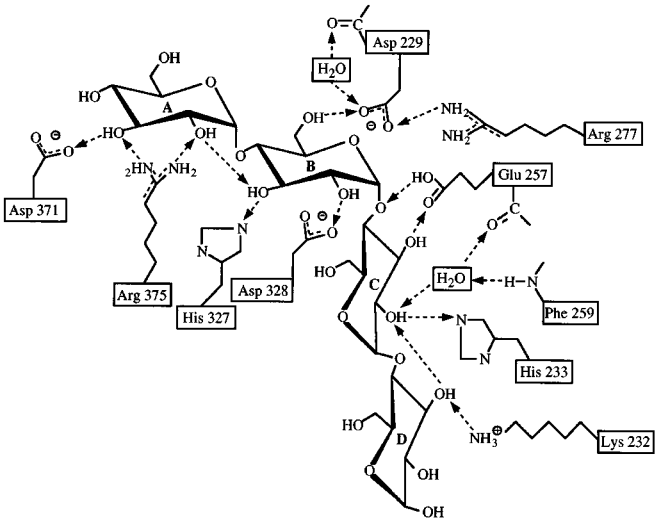

FIG. 4. **Schematic representation of the interactions between maltotetraose and CGTase active site residues.** Because acarbose has a better quality electron density complexed with CGTase but deviates structurally from natural substrates at the A and B sugars, the contacts shown are a combination of the contacts observed in the structures of CGTase complexed with maltotetraose and acarbose.

The α-CD at maltose-binding site 1 is involved in crystal packing contacts that result in the formation of two additional weak hydrogen bonds between residues Ser-385 and Arg-339 of a symmetry related molecule and sugars C and D of α-CD. Besides the stacking of this α-CD onto the indole groups of Trp-616 and Trp-662, no stacking of glucoses of the α-CD ring onto hydrophobic residues of neighboring protein molecules is observed. In conclusion, the energetic contribution of crystal packing contacts on α-CD binding seems to be low. Only weak electron density is observed between the Trp-616 carbonyl oxygen and the O-6A and O-5A hydroxyls of glucose A, whereas in the native protein structure (2) a water molecule was observed to mediate hydrogen bonds between maltose and CGTase. Possibly, the lower resolution of the double mutant structure causes this water molecule not to be observed. Because flash freezing of the crystal reduced the unit cell volume by about 3–4% (*cf.* Table I), some contacts at maltose-binding site 1 could be altered by small changes in crystal packing.

Maltose-binding site 2 is not involved in crystal packing contacts. The α-CD that binds at this site stacks onto the aromatic ring of Tyr-663. In addition, the side chain of Leu-600 protrudes into the cyclodextrin ring as illustrated in Fig. 5. The Leu-600 side chain methyl groups approach the apolar surfaces of sugars C and D of the bound α-cyclodextrin to a distance of about 4–4.5 Å. A similar mode of binding was observed in the structures of pig pancreatic α-amylase (37) and soybean β-amylase (38), where a valine and a leucine residue, respectively,

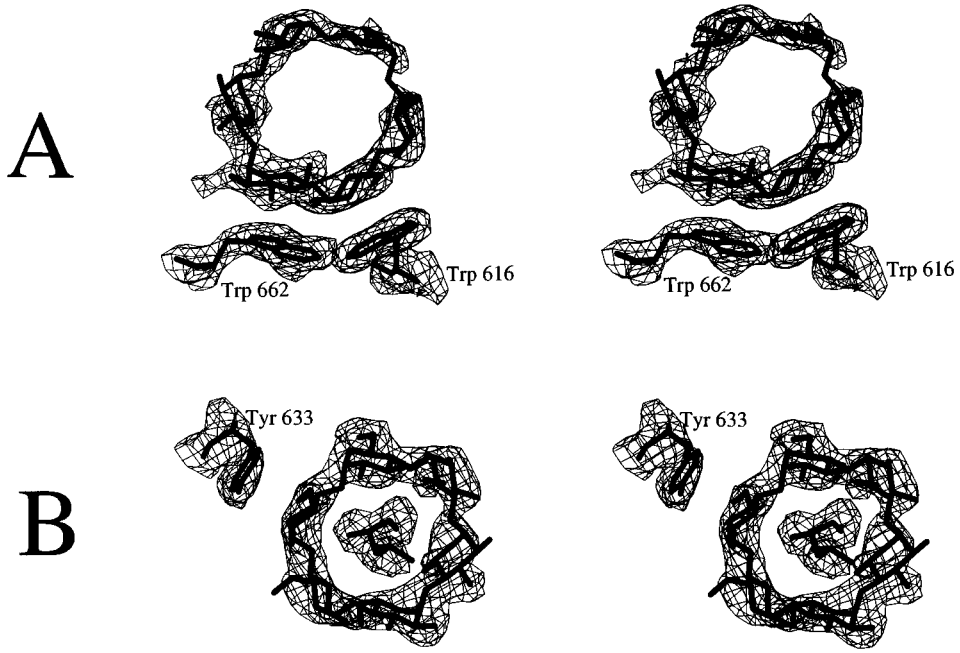

FIG. 5. **Stereo views of α-cyclodextrin bound at maltose-binding sites 1 and 2 in the E-domain of CGTase.** Shown are α-CD bound to maltose-binding site 1 (*A*) and α-CD bound to maltose-binding site 2 (*B*). Electron density in $2F_o - F_c$ maps is contoured at 1 σ, and hydrophobic residues of the protein involved in stacking interactions with the oligosaccharides are indicated. The residue in the *middle* of the cyclodextrin ring in *B* is Leu-600.

TABLE IV
Possible intermolecular hydrogen bonds between D229N/E257Q CGTase and α-cyclodextrin

The contacts between wild-type CGTase and maltose are shown for reference. The nomenclature of the maltose binding sites is as proposed by Lawson *et al.* (2).

α-Cyclodextrin residue/atom	Protein atom or water molecule	D229N/E257Q CGTase distance	Wild-type CGTase distance
		Å	Å
Maltose-binding site 1			
Residue A			
O-2A	Asn-667 Oδ1	2.7	2.8
O-3A	Asn-667 Nδ2	2.8	3.2
O-5A	Water/W616 O		3.4
O-6A	Water/W616 O		2.7
Residue B			
O-2B	Water	2.7	3.3
O-3B	Lys-651 Nζ	2.8	3.2
O-5B	Water/S382 OH	2.8	2.5
O-6B	Water/S382 OH	3.5	>3.5
Residue C			
O-5C	Water	2.9	
O-6C	Ser-384 O	3.4	
Residue D			
O-6D	Water	2.8	
O-6D	Arg-339 Nη-1	3.3	
Maltose-binding site 2			
Residue A			
O-2A	Ala-599 O	2.7	3.2
O-2A	Gly-601 N	2.8	2.9
O-3A	Water		2.9
O-3A	Gly-601 N	2.7	>3.5
O-6A	Asn-627 Oδ-1	2.7	3.1
O-6A	Gln-628 Nε-2	3.4	3.4
Residue B			
O-2B	Thr-598 O	3.3	2.8
O-3B	Ala-599 O	3.1	2.6
O-3B	Thr-598 Oγ-1	3.1	2.8
Residue E			
O-3E	Water	3.4	

were observed to protrude into the ring of a bound cyclodextrin molecule. The water-mediated contact between Asn-603 and maltose that was observed in the native protein is not substantiated by electron density in the double mutant structure. The role of this water molecule appears to be taken over by the main chain amino group of Gly-601, which is at hydrogen bond distance of the O-2A and O-3A hydroxyl groups of α-CD.

DISCUSSION

The active site mutant proteins D229N, D328N, E257Q, and D229N/E257Q still displayed cyclodextrin-forming activity, although their activities were very low compared with wild-type CGTase. The activities could not be measured in culture supernatants but only in purified and concentrated protein samples. These results are in agreement with those of Klein *et al.* (7), who described low activities for the D229A and D328A mutations in *B. circulans* strain 8 CGTase. They disagree with the results obtained with a CGTase from an alkalophilic *Bacillus* (39), where no detectable activity could be measured for the same active site mutations as described in this report. Analogous mutations in the active site residues of *Bacillus stearothermophilus* Neopullulanase (40) and *Aspergillus oryzae* Taka-amylase A (41) also showed no detectable activities. However, the former two proteins were not purified and concentrated before measurement.

The E257Q and D328N mutants display a negative shift of 0.5 pH units of their pH optima compared with that of the wild type (pH 6.0). Glu-257 is thought to donate a proton to the glycosidic bond during catalysis, and Asp-328 forms a hydrogen bond with Glu-257 in the unliganded structure, in this way elevating the pK_a of this glutamate. In the D328N mutant this is no longer possible, which explains the observed decrease of the pH optimum of CGTase. The E257Q mutant displays residual activity as well and has also a decreased pH optimum. Even the D229N/E257Q double mutant CGTase is still catalytically functional as is demonstrated by the presence in the active site of maltotetraose, which is a degradation product of α-cyclodextrin. Possibly, the third acidic active site residue, Asp-328, partially takes over the roles of Asp-229 and Glu-257, even though it is located somewhat more remote from the glycosidic bond to be cleaved. The pK_a of Asp-328 is likely to be affected by a E257Q mutation, and this may explain the shift in pH optimum observed for the E257Q mutant. Site-directed mutagenesis of each of these three catalytic site residues yields a protein with greatly reduced activity, demonstrating that their function in catalysis is highly correlated. Mutation D229N displays no effect on the pH optimum, which suggests that this residue does not affect proton transfer. It is in agreement with the proposal that Asp-229 acts as a general base or nucleophile near the C-1 atom of the sugar residue at subsite B (3).

The C-6 hydroxyl group at sugar residue B is absent in acarbose but present in maltotetraose. This functional group was suggested to be essential for a contact with His-140 that could induce a strained conformation in sugar B prior to breaking of the scissile glycosidic bond (3). The Asp-229 residue could assist such an arrangement by hydrogen bonding to the C-6 hydroxyl group. Alternatively, this hydroxyl group might polarize either the O-5 or the C-1 atom of its own sugar in order to activate the scissile bond. In the structures reported here, the C-6 hydroxyl group is at about 3 Å from the Cϵ-1 atom of the highly conserved His-140 (42), which retains a conformation similar to that in the unliganded protein. The Asp-229 Oδ-2 atom forms a strong hydrogen bond with the C-6B hydroxyl group and places it above the plane of the O-5 and C-1 atoms of its own sugar, whereas the Asp-229 Oδ1 atom is positioned close to the C-1B atom. Such a configuration could well distort the sugar conformation and polarize the scissile bond between sugars B and C prior to catalysis. The distorted electron density observed at subsite B suggests that the conformation of this sugar is indeed destabilized by these contacts, similar to what was observed in lysozyme-carbohydrate complexes (43–45).

The question remains why maltotetraose is not cleaved by CGTase, even though it has the C-6 hydroxyl group present at subsite B and the enzyme is still catalytically functional at pH 9. The lower quality of the electron density observed for maltotetraose, relative to that observed for acarbose (3), suggests that acarbose is a better inhibitor than maltotetraose. This could be attributed to acarbose lacking the C-6 hydroxyl group. In the case of maltotetraose there is apparently an additional factor that prohibits its cleavage. From biochemical studies it is known that CGTase does not interconvert mixtures of glucose, maltose, maltotriose, and maltotetraose (14) and that maltotetraose is the strongest cyclization inhibiting substrate among saccharides consisting of one to seven glucose residues in the case of a β-CGTase from an alkalophilic *Bacillus sp.* (ATCC 21783) (46). Apparently efficient catalysis is not solely determined by the exact composition of the oligosaccharide but also by the size of the sugar polymer. Whether CGTase cannot perform catalysis because certain contacts with sugars beyond subsites A and D are lacking requires structural data on longer substrates complexed with CGTase.

The presence of two α-cyclodextrin molecules in the double mutant structure at maltose-binding sites 1 and 2 demonstrates that cyclodextrins are capable of binding strongly to the

E-domain. Especially the cyclodextrin bound to the maltose-binding site near Tyr-633 could interfere with the catalytic activity of the enzyme because this maltose-binding site is part of the long groove on the protein surface leading into the active site (2). When the product concentration increases in time, it is clear that cyclodextrins can compete with raw starch in binding to the E-domain and thus may block the binding of linear starch polymers and the interaction with raw starch granules.[2] It is interesting to find a hydrophobic amino acid, Leu-600, exposed to the solvent at the second maltose-binding site. Its involvement in binding an α-CD molecule by protruding with its side chain into the cyclodextrin ring, suggests that this site on the protein is intended to bind cyclic or helical polysaccharides like cyclodextrins and amylose. It should be noted, however, that Leu-600 appears to make only weak van der Waals contacts with the α-CD and could function only to position the substrate correctly. Maltose-binding site 1 is thought to attach the protein nonspecifically to the raw starch granules; thus the enzyme could also be inhibited by binding of its products to this site.

The crystal structures of CGTase complexed with maltotetraose and α-cyclodextrin presented here demonstrate clearly that product inhibition and enzymatic functionality are tightly interwoven. Because the cyclodextrin molecules basically bind with only two glucose residues to the maltose-binding sites, mutations that will specifically prevent the binding of cyclic products are not immediately obvious. Mutation of Leu-600 and adjacent residues could reduce the binding of cyclic products to maltose-binding site 2 while the binding of linear substrates remains unaffected.

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Crystallographic Studies of the Interaction of Cyclodextrin Glycosyltransferase from *Bacillus circulans* Strain 251 with Natural Substrates and Products

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